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Standard Guide for the 'Salmonella typhimurium'/Mammalian Microsome Tests for Bacterial Mutagenicity

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Research Triangle Park, NC

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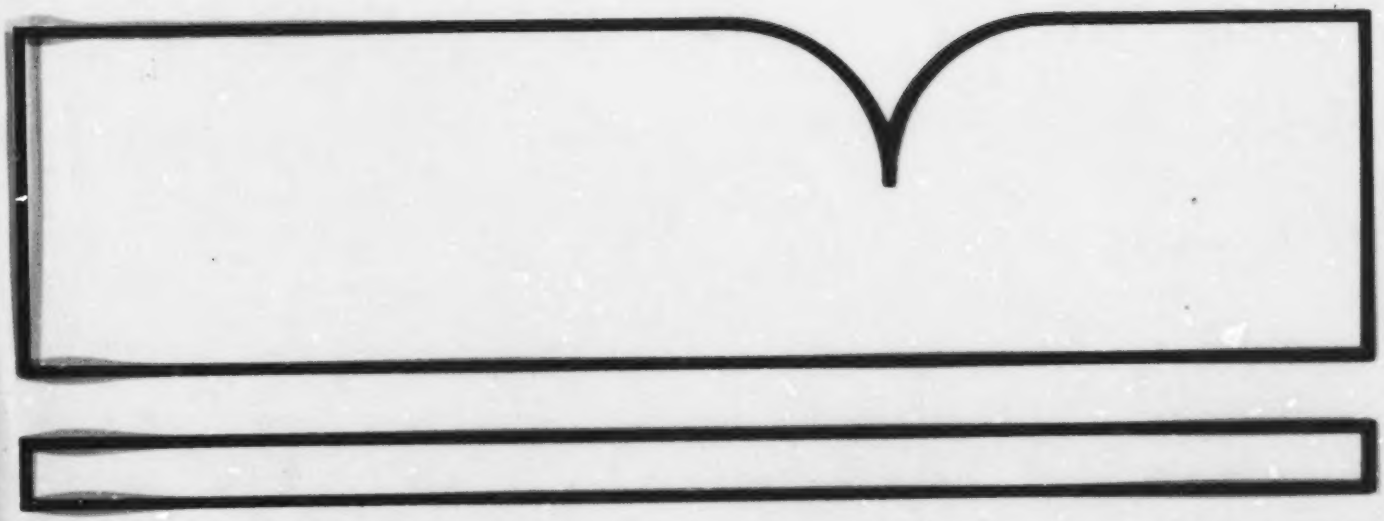

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August 1986

A STANDARD GUIDE FOR THE SALMONELLA TYPHIMURIUM/MAMMALIAN MICROSOME TESTS FOR BACTERIAL MUTAGENICITY

The panel members were L.D. Claxton (Chairman), Jane Allen, Angela Auletta, Joyce McCann, Kristien Mortelmans, Earle Nestmann, and Errol Zeiger.

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16. ABSTRACT

Since its development by Dr. Bruce Ames and his coworkers, the Salmonella typhimurium/mammalian microsome mutagenicity assay has been used widely throughout the world. Many authors have suggested various modifications and made recommendations in regards to this assay. Although the recommendations of a panel of experts was published in 1979 by deSerres and Shelby, a committee of members of the Environmental Mutagen Society (EMS) initiated this effort in response to the encouragement by the American Society of Testing and Materials (Committe E47.09.01) and because of new developments within the field of microbial mutagenesis tests, but it is not intended for these recommendations to replace or diminish the usefulness of presently available protocols and procedures.

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A GUIDE FOR THE SALMONELLA TYPHIMURIUM/MAMMALIAN MICROSOME TESTS FOR BACTERIAL MUTAGENICITY

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A GUIDE FOR THE *SALMONELLA TYPHIMURIUM*/MAMMALIAN MICROSOME TESTS FOR BACTERIAL MUTAGENICITY

1. INTRODUCTION

This guide was developed by a joint committee of American Society for Testing & Materials (ASTM) and the Environmental Mutagen Society (EMS) members in order to aid toxicologists, geneticists, biochemists, other researchers and interested persons in the understanding, performing, and analyzing of mutagenicity tests that use strains of the bacterium *Salmonella typhimurium*. Although the principles set forth in this report consider primarily the plate incorporation test, they can be applied, with some modification, to other protocol methods (spot test, preincubation, etc.) and also to testing with bacteria other than Salmonella. In this rapidly changing area of toxicology, it is not intended for these recommendations to replace, alter, or diminish the usefulness of presently available protocols and procedures. Its purpose, instead, is to provide a guide for people who perform or evaluate microbial mutagenesis tests in accomplishing the following goals:

1.1 provide an understanding of the critical procedures (steps) involved in bacterial mutagenicity testing,

1.2 provide general criteria by which researchers can evaluate whether they are performing and utilizing these test systems properly,

1.3 provide criteria by which individuals who are responsible for the evaluation of experimental *Salmonella typhimurium* mutagenicity data can determine whether or not tests have been performed properly, and

1.4 provide a basis by which new procedures and developments in bacterial mutagenicity testing procedures can be evaluated.

2. SCOPE

2.1 As stated previously, the purpose of this guide is to establish criteria by which protocols and procedures for the *Salmonella typhimurium* plate incorporation mutagenicity test can be evaluated. These guidelines are also intended to aid laboratory personnel and others in gaining a better understanding of the critical elements involved in bacterial mutagenicity testing.

2.2 The scope of this document is limited to procedures used solely for the testing of substances to determine mutagenicity and does not apply to other methods and uses such as exploring mechanisms of mutation. It also is not the intent of this document to discuss the relationships between bacterial mutagenicity testing results and the results observed in other tests for mutagenicity and carcinogenicity.

2.3 Since all protocols for bacterial mutagenicity testing have certain critical elements in common, the general principles contained in this document can also be applied to other test systems.

3.0 SIGNIFICANCE AND USE

This guide is limited to procedures used solely for the testing of substances to determine their mutagenicity and does not apply to other methods and uses such as exploring mechanisms of mutation.

4.0 APPLICABLE DOCUMENTS

ASTM Standards: None are known.

5.0 TEST METHOD

5.1 *Principle:* The Salmonella reverse mutation assay is based on the use of several selected *Salmonella typhimurium* strains that revert from histidine dependence (auxotrophy) to histidine independence (prototrophy) at an increased rate in the presence of a mutagen. The test detects a wide range of mutagens, including many that require an exogenous metabolic activation system. The test is used as a screen for the mutagenic

activity of pure compounds, complex mixtures, component fractions, and body fluids.

5.2 *Description*

5.2.1. Tester strains: At present the most commonly used Salmonella strains are TA1535, TA1537, TA1538, TA98 and TA100 (Ames, 1979; Ames, et al., 1975; Maron and Ames, 1983; deSerres and Shelby, 1979). The number and type of strains used depend upon the availability and type of sample, the focus of the study, and previous knowledge concerning the test material. In addition to having a mutation in one of the genes of the histidine operon, tester strains frequently have mutations that impart other specific characteristics to the tester strain. One mutation (*rfa*) leads to a defective lipopolysaccharide coat; another is a deletion of genes involved in the synthesis of the vitamin biotin (*bio*) and in the excision repair of DNA damage (*uvr*B). The *rfa* mutation increases the permeability of the strains to large molecules, thereby increasing the mutagenicity and/or toxicity of these chemicals. The *uvr*B mutation leads to a reduced level of error-free repair of some types of DNA damage and thereby enhances the strains' sensitivity to certain chemical and physical mutagens. Strain TA1535 carries the *his*G46 mutation and is reverted by mutagens that produce base-pair substitutions. Strain TA100 is derived from TA1535 by the introduction of the plasmid pKM101 which increases the sensitivity of mutagen detection by enhancing error-prone DNA repair. The presence of this plasmid makes TA1OO respond to some frameshift mutagens as well as base pair substitution mutagens. Strain TA1537 carries the frameshift mutation *his*C3076, and strain TA1538 carries a different frameshift mutation, *his*D3052. These two mutations are reverted by different classes of frameshift mutagens. Strain TA98 is derived from TA1538 by the introduction of plasmid pKM101.

Other Salmonella strains related to these five strains, but with different characteristics in terms of DNA repair capacity, cell permeability, and the presence of plasmid pKM101 also are available and have been described by Ames et al. (1975). Three strains, TA97, TA102, and TA104, recently have been developed and described in detail

(Levin, et al., 1982a,b). Strain TA97 responds to frameshift mutagens and was recommended to replace strain TA1537 (Maron and Ames, 1983). Strain TA102 and TA104 were developed to detect base-pair substitution mutagens not detected by TA1535 and TA100.

All tester strains should be maintained and stored according to published methods (Ames, et al., 1975; Maron and Ames, 1983; deSerres and Shelby, 1979). They should be analyzed on a frequent and rational basis for each characteristic needed for strain identification or for characteristics that could affect the test. For example, strain identification could include the following:

- histidine and biotin requirement
- UV sensitivity (presence of the *uvr*B deletion)
- crystal violet sensitivity (presence of the *rfa* mutation)
- ampicillin and/or tetracycline resistance (presence of the the appropriate plasmid)
- spontaneous reversion frequency, and
- reversion characteristics to various positive controls.

The spontaneous reversion frequency may vary considerably between different laboratories and even within the same laboratory over time. Each laboratory should use not only published ranges of response but also its own historical data base, with self-imposed limits for determining acceptable spontaneous reversion counts for positive and negative controls. The presence of the metabolic activation system may increase or reduce the spontaneous reversion counts of certain strains.

5.2.2. Exogenous metabolic activation mixture: Metabolic conversion of promutagens to mutagens usually is accomplished using an exogenous metabolic system consisting of a tissue homogenate and cofactor mix. The tissue homogenate used most often is a 9000 xg supernatant (S9) of rat liver. The homogenate and cofactor mixture should be prepared in accordance with published methods (Ames, et al., 1975; Maron and Ames, 1983) or should be described in detail in any resulting publication or report. The rationale for

using alternative methods of metabolic activation must be justified (see Section entitled Test Report). Different compounds have different optimum requirements for activating enzymes, and various concentrations of the exogenous activation system may be used in a given test. Although concentrations (ml S9/ml S9 mix) as low as 1% and as high as 50% have been reported, a range of 4 to 30% is more common. The selection of animals and tissues, induced or uninduced, for the preparation of the S9 fraction usually is made by the investigator. For general screening purposes, however, the S9 fraction most ofter used is obtained from Aroclor 1254-induced male rat or hamster livers. It is important that all activation mixtures be checked using positive control mutagens that require this exogenous activation. All reports should specify the amount and concentration of activation mixture used.

5.2.3. General Testing Procedure: Although a variety of procedures can be used, the following provides a description of the plate test as generally performed (Maron and Ames, 1983).

To 2-3 ml of top agar (maintained at about 43°C) is added:

- approximately 10^8 bacteria from a fresh culture
- approximately 0.5 ml metabolic activation mixture or buffer
- between 0.05 ml and 0.1 ml of a solution of test compound or solvent.

This mixture is mixed gently and poured on plates that contain about 25 ml of minimal-glucose agar. After the top agar has solidified, the plates are inverted and incubated at 37°C for 48 hours or 72 hours. The plates then are scored for the number of revertant colonies and for the presence of a background lawn of auxotrophic bacteria.

When it is necessary to determine a suitable nontoxic dose range, a preliminary range-finding test usually is conducted over a wide range of dose levels using one tester strain. Final testing is performed with a suitable battery of strains in the presence and the absence of a mammalian metabolic activation system using several dose levels. The highest dose used will depend on the solubility and/or toxicity of the test compound, and

ideally, only the highest dose level should be toxic to the bacteria. Toxicity of the solvent also should be considered in the selection of doses used. Two to three plates per dose level usually are used, and repeat assays are strongly recommended to demonstrate reproducibility.

Some investigators prefer to use modifications of the plate incorporation procedure. For the preincubation test (Yahagi, et al., 1975; Maron and Ames, 1983), the test compound, bacteria and the S9 mix or buffer, are incubated at 25°C or 37°C, usually for about 20 minutes, before adding approximately 2 ml of top agar. All other procedures are the same as for the plate incorporation assay. Other modifications of the test system that have been published include methods for volatile chemicals, reductive metabolism, conjugated mutagens, and simple spot tests (Zeiger, 1985).

6.0 TEST MATERIALS

6.1 *Reagents*

6.1.1. Metabolic Activation Preparations: Preparations may be prepared from a variety of sources depending on the needs of a particular assay. Factors which may vary include, for example, species of animal or plant, sex, tissue, age, method of induction (Aroclor 1254, phenobarbitol, etc.), and method of preparation (eg, S9 versus S12). Co-factor mixes for enzyme preparations should be shown to support enzyme activity, as measured either directly or by a biological effect. It is important to demonstrate that the metabolic preparation used converts appropriate promutagens to mutagens.

6.2 *Media*

The following criteria should be observed closely:

· Medium in which cultures are grown before mutagenesis treatment should support growth such that an adequate and reproducible number of viable cells are available for treatment.

· Selective medium (i.e. medium that will support growth only of bacteria that have undergone mutation) should be used which allows complete bacterial growth whenever the selecting agent is either removed (e.g., 8-azaguanine) or added (e.g., histidine).

· Care should be taken that supplies do not affect experimental results. For example, ethylene oxide-sterilized plasticware should not be used under conditions where residual ethylene oxide may be present, because it is mutagenic to strains TA100 and TA1535. Similarly, glass pipets should be used with compounds or solvents which react with plastic pipets or the dyes used to calibrate them. In other words, neither reagents, media, nor supplies should substantially affect either the spontaneous number of revertants nor the number of induced revertants.

6.3 *Reference Substances*

Presently, no reference substances are mandatory for use as concurrent positive and/or negative controls. Researchers should consult published protocols (Maron and Ames, 1983) for chemicals that can be utilized in this manner. When possible, one set of controls should have chemical properties and/or structures similar to the substances being tested.

7.0 TEST DATA

7.1 *Treatment of Results*

7.1.1. Gathering data (counting plates): After a suitable incubation period (48-72 hours), the petri dishes are examined and the colonies counted. Within the nontoxic dose range, the plates should have an uninterrupted background lawn of parental cells (microcolonies) with an evenly distributed number of overgrowing mutant colonies (Maron and Ames, 1983). The mutants may be counted manually or with an automatic colony counter. The advantages of using an automatic counter include speed and the possibility of a direct interface with a computer or data acquisition system. With proper adjustment and periodic recalibration, automatic counting compares favorably with manual counting (Claxton, et al., 1984); it is more efficient and is without the disadvantage of scorer fatigue.

7.1.2. Entering data. Individual plate counts must be recorded in laboratory notebooks, on tape or disc, etc. so that a permanent record of the results is available. Means and standard deviations (or standard errors) should be calculated for replicate plate counts at each dose, and concurrent control data should be added to the historical control data base. Disruptions of the lawn, as well as contamination or test substance precipitation, should be noted.

7.1.3. Plotting data (optional). When test data are clearly negative, graphical representation usually is not useful and ordinarily will not be done, except in cases in which plots are produced automatically as part of the "hard" printed copy of the results. Visualization of data in graphical form often aids in their interpretation, so that manual or computer-directed plotting of results is a useful step.

7.2 *Statistical Evaluation*

To paraphrase McCann et al. (1984), many test results are clearly negative or positive, and statistical analysis is not needed to provide this answer. What is needed by investigators using the test is a consistent and acceptable method for analyzing experiments, especially those with questionable results. While the widely accepted "2-fold" or "modified 2-fold" rules (that include consideration of dose responsiveness) compare favorably with statistical rules (Chu, et al., 1981), this approach does not take into account the differences in spontaneous backgrounds of the various tester strains, the plate-to-plate variability, and other statistical considerations (McCann, et al., 1984). An empirical approach based on the linear portion of dose response curves has been proposed to serve as an adequate and sensitive statistical analysis (McCann, et al., 1984; Bernstein, et al., 1982) although other models have been proposed (Moore and Felton, 1983; Stead, et al., 1981; Margolin, et al., 1981; Myers, et al., 1981). Investigators are encouraged to be familiar with a number of statistical methods and the assumptions and rationales for the use of each so that appropriate methods can be incorporated where needed. Certain of the various models that have been proposed in the literature (Bernstein, et al., 1982; Stead, et al., 1981; Margolin, et al., 1981; Snee and Irr, 1984) have been reviewed recently (Broekhoven and Nestmann, 1986). The models are based on slightly different distributions and assumptions, but Broekhoven and Nestmann concluded that any one of them is reasonable (Broekhoven and Nestmann, 1986).

The majority of Salmonella test data in the literature have not been evaluated using formal statistics. In general, decisions of "positive" and "negative" are made on an ad hoc (best judgment) basis,and many reports in the literature appear to rely on a strict "two-fold increase only" rule. This rule states that if a doubling over background of *His+* revertants is seen, the chemical is considered mutagenic (the number of doses or plates used is not a factor in this evaluation). However, the reference, that often is cited as the source of this rule (Ames et al., 1975), discussed the need for a reproducible dose-related

response. Use of this "two-fold only" rule can lead to false conclusions because it does not require a dose-related response; therefore, a chemical can be called a mutagen if it causes a doubling of mutants, at one dose only, and called a nonmutagen if a reproducible dose-related increase falls short of two times the background. Strict interpretation of this rule does not require that the effect be reproducible and also ignores the magnitude of the response, giving as much weight to an increase from 10 colonies to 20 colonies per plate, as an increase from 100 to 200 colonies per plate, regardless of the variation around the average counts.

7.3 *Interpretation of Results*

It is clear that regardless of the statistical procedure used, the results must meet a number of criteria, such as derivation from a defined protocol covering a range of doses and reproducibility. The anticipated or known carcinogenicity or noncarcinogenicity of the chemical must not be a consideration in the evaluation of the data.

All positive and negative control data must meet expected and accepted values. If the data do not meet the statistical criteria for a positive result, and there are no "hints" of positive activity that fail to meet the strict evaluation criteria, then the experiment probably is negative (see requirements for a non-mutagen below). Tests may show slight increases of activity, for example, in only one strain or with a certain amount of S9, often at only one dose level. In these cases, efforts should be made to expand the protocol to include differing amounts of S9, to expand the dose scale around the dose in question, or to provide other protocol modifications, in an attempt to clear any doubt about the validity of the results. If further testing does not clarify whether or not the substance is mutagenic, the result is said to be either questionable or indefinite.

7.3.1. Requirements for a mutagenic response: In the absence of a valid statistical procedure, as defined above, the most appropriate determinant of a positive (mutagenic) response is a reproducible, dose-related increase in mutant colonies. No evidence

supports a specific requirement for a 2- or 3-fold increase over background. In the evaluation of test results, consideration should be given to plate-to-plate variability. The larger this measure of variability, the larger the increase over background needed to declare a chemical mutagenic. If the experimenter does not feel confident in calling a less-than-two-fold increase over background positive, the conclusion should be "equivocal."

7.3.2. Requirements for a nonmutagenic response: The declaration of a negative (nonmutagenic) conclusion requires a heavier burden of experimentation than the declaration of a positive. Before a test sample is declared negative (or nonmutagenic) it must have been tested both with and without exogenous metabolic activation in replicate plates, at multiple doses, up to a maximum dose of 5 to 10 mg per plate, unless limited by solubility or toxicity. If a toxic effect is seen, as determined by a clearing of the background lawn or a decrease in the number of revertant colonies on the plate, the lower doses tested should show no evidence of toxicity. Concurrent solvent and positive controls should be present. The solvent control should be within the historical range for the laboratory and should be consistent with the values either published by the investigator(s) who developed the tester strain or established through interlaboratory studies. Positive controls should demonstrate a mutagenic response for each Salmonella tester strain used and also should demonstrate that the exogenous activation mix was active. Preferably, the dose of the positive control should be approximately midrange of the linearly increasing dose response curve for that positive control.

No clear-cut recommendation specifies the number or battery of tester strains which should be used prior to the declaration of a negative response. In the past, the strains TA98, TA100, TA1535, and TA1537 were considered sufficient, although some laboratories also recommended the use of TA1538. Recently, Levin, et al. (1982a,b) have reported the construction of new tester strains (TA97, TA102, and TA104) designed to be more sensitive than some of the earlier strains and extending the range of chemicals

which can be detected. Although each strain will detect a different group of mutagens, and some mutagens may be active in only one strain, it is not necessary to use all 8 tester strains (plus others not mentioned here) before declaring a chemical to be negative. The experimenter's best judgment should be exercised and justified when selecting the strains used.

When it is known that a specific class of chemicals is active in a specific tester strain or has optimal mutagenic activity when tested with a metabolic activation system or protocol other than the laboratory's standard protocol, the other system should be used by itself or in parallel with the standard protocol when testing related chemicals. If this is not done, the negative results should carry the caveat that the test may not have been performed under the optimum conditions for detecting the mutagenicity of the test substance.

If insufficient test sample is available for an adequate test, as defined above, the investigator should make careful and justifiable choices as to the strains, conditions, etc. chosen.

8.0 TEST REPORT

8.1 *General*

The test report, whether for submission for publication in the open literature (eg, journal) or to a regulatory agency, should be as complete as possible and should contain: 1) enough detail to assure the reviewer that the test was performed according to accepted procedures and 2) sufficient information (including data) to allow verification of the results. Transformed data alone are not acceptable. Reports submitted to regulatory agenices will, ordinarily, contain more detailed information about the test procedure and chemical identity and composition than reports submitted for publication. Certain critical elements, which should be considered by the investigator when preparing

submissions, are set forth below.

8.2 *Title*

The title should be as complete as possible and should reflect accurately the contents of the report.

8.3 *Test Agent*

Details of the identity, purity, and other characteristics of the test substance should include, as appropriate, CAS registry number, commonly used synonyms, molecular structure, source, lot number, method of analysis for purity (if performed), percent of impurities, physical state, and stability. The interval between receipt of the chemical, preparation of solutions, and use, should be given with details about storage conditions, and any other information that could be considered relevant.

8.4 *Test System*

The term "Ames assay" is an incomplete descriptor which can include several different strains and test procedures. Such generic designations should be clarified by a complete description of the test system used. The test organism(s) should be clearly identified. Source of the organisms used should be specified along with storage and growth conditions, and methods of phenotypic characterization.

8.5 *Procedures*

When writing for submission to a journal or other "open-literature" publication, the author should bear in mind that the aim of a Methods section is to permit repetition of the test conditions and to insure reproducibility of the reported results. If possible, methods should be reported as concisely as possible with references to well established procedures in seminal papers, not in secondary sources. Modifications of referenced protocols should be detailed.

When preparing a report for submission to a regulatory agency, the procedures usually are required to be in even greater detail. When a published guideline, such as this guideline, is being followed, this should be stated clearly and referenced. Modifications to the guidelines, to the stated protocol or to any relevant laboratory standard operating procedures (SOPs) should be documented and the reasons for such deviations should be stated clearly. In preparing a report for submission to a regulatory agency, the author should bear in mind that the aim of this section is verification that the test was performed according to acceptable methods., any information relevant to this determination should be presented.

The Methods section should include the number of concentrations tested, the rationale for dose selection, toxicity information, and information on factors which may affect the results. The latter may include, but is not limited to, solvent, pH, temperature of incubation, preincubation conditions, composition of the media, artificial lighting conditions, length of incubation, and time and temperature of storage between the end of the incubation period and determination of the results. The number of replicates, both within an experiment (e.g., number of plates per point) and of the entire experiment, should be indicated. Positive and negative control data should be presented. Details of the metabolic activation system used should be presented. This may include but is not limited to suppliers (e.g.,of S9), animal species, organs, enzyme inducers, method of preparation, storage conditions, procedures followed during use, composition of the cofactor cocktail and enzyme characterization procedures. Again, the author should present any information relevant to evaluation of the study design and interpretation of the reported results.

8.6 *Results*

Primary data sufficient to verify the results should be presented even if graphic presentations are included. Transformed data should not be presented unless the method of transformation is clearly defined and sufficient primary data are presented to permit

an independent evaluation of the data.

8.7 *Data Evaluation*

The author should state clearly the criteria for data evaluation and should apply appropriate statistical methodology where applicable.

8.8 *Study Acceptability*

For regulatory purposes, the criteria for acceptability of a study should be clearly stated. Any studies which are not acceptable by the author's criteria should be cited and the reason for rejection given.

8.9 *Good Laboratory Practices (GLP's)*

A report to a regulatory agency should reference any GLPs followed. This also should include a statement of quality assurance, audit procedures, and any relevant findings.

8.10 *Conclusion*

The author's conclusion should be clear and concise. Authors should consider relating the results and information gained to results previously reported for the same or similar substance and should, when appropriate, discuss associated factors such as metabolism, structure-activity relationships, chemistry, etc.

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